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Abstract: Alzheimer's disease (AD) is a genetically complex disorder, and several genes related to cholesterol metabolism have been reported to contribute to AD risk. To identify further AD susceptibility genes, we have screened genes that map to chromosomal regions with high logarithm of the odds scores for AD in full genome scans and are related to cholesterol metabolism. In a European screening sample of 115 sporadic AD patients and 191 healthy control subjects, we analyzed single nucleotide polymorphisms in 28 cholesterol-related genes for association with AD. The genes HMGCS2, FDPS, RAFTLIN, ACAD8, NPC2, and ABCG1 were associated with AD at a significance level of $P \leq 0.05$ in this sample. Replication trials in five independent European samples detected associations of variants within HMGCS2, FDPS, NPC2, or ABCG1 with AD in some samples ($P = 0.05$ to $P = 0.005$). We did not identify a marker that was significantly associated with AD in the pooled sample ($n = 2864$). Stratification of this sample revealed an APOE-dependent association of HMGCS2 with AD ($P = 0.004$). We conclude that genetic variants investigated in this study may be associated with a moderate modification of the risk for AD in some samples.

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Association study of cholesterol-related genes in Alzheimer's disease

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To identify further AD susceptibility genes, we have screened genes that map to chromosomal regions with high logarithm of the odds scores for AD in full genome scans and are related to cholesterol metabolism. In a European screening sample of 115 sporadic AD patients and 191 healthy control subjects, we analyzed single nucleotide

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polymorphisms in 28 cholesterol-related genes for association with AD. The genes *HMGCS2*, *FDPS*, *RAFTLIN*, *ACAD8*, *NPC2*, and *ABCG1* were associated with AD at a significance level of $P \leq 0.05$ in this sample. Replication trials in five independent European samples detected associations of variants within *HMGCS2*, *FDPS*, *NPC2*, or *ABCG1* with AD in some samples ($P=0.05$ to $P=0.005$). We did not identify a marker that was significantly associated with AD in the pooled sample ($n=2864$). Stratification of this sample revealed an *APOE*-dependent association of *HMGCS2* with AD ($P=0.004$). We conclude that genetic variants investigated in this study may be associated with a moderate modification of the risk for AD in some samples.

Keywords *HMGCS2* · *FDPS* · *NPC2* · *ABCG1* · Polymorphism

Introduction

Cholesterol influences processes that are central to the pathogenesis of Alzheimer's disease (AD) [1]. In support of experimental and epidemiological evidence, population genetics suggest a role of cholesterol in the pathophysiology of AD [2]. The $\epsilon 4$ allele of *APOE*, the gene encoding the major apolipoprotein of the central nervous system is the only well-established genetic risk factor for sporadic AD. However, several other genes related to cholesterol metabolism have been associated with AD risk, albeit with conflicting findings in subsequent replication trials (The AlzGene Database, <http://www.alzgene.org>). With respect to the complex nature of the genetics of both cholesterol metabolism and AD, we have recently conducted a set association study in which we have shown an additive effect of polymorphisms in cholesterol-related genes for which association with AD risk had been published in previous single gene studies [3]. Against this background, we investigated further genes from the same functional context for association with the risk for AD in a targeted screen of cholesterol-related genes. Full genome scans have identified chromosomal regions with high logarithm of the

odds (LOD) scores for AD which supposedly harbor AD susceptibility genes [4, 5]. Because positional candidate gene selection might reduce the prior probability of false positive associations, we confined our screen to AD-linked chromosomal regions [6].

In this paper, we report that of 28 investigated genes, *HMGCS2*, *FDPS*, *RAFTLIN*, *ACAD8*, *NPC2*, and *ABCG1* were associated with the risk for sporadic AD in a small screening sample. However, none of the observed associations could be consistently replicated.

Materials and methods

Patients and control subjects

Case control samples for sporadic AD from six independent clinical centers in Switzerland, Germany, Poland, Belgium, Sweden, and Greece were included in genetic association studies. Clinical diagnosis of probable AD was made according to the NINCDS-ADRDA criteria [7]. Dementia and memory deficits in geographically matched control subjects were excluded by neuropsychological testing, consisting of the "Consortium to Establish a Registry for Alzheimer's Disease" (CERAD) neuropsychological test battery and the "mini mental status examination" (MMSE). Cases and controls from the German series were histopathologically confirmed. The local Ethics Committees approved of the study, and informed consent was obtained before the investigation. Table 1 summarizes the sample characteristics. Screening was done in a random subsample of the Swiss series comprising 115 AD cases and 191 healthy control subjects (HCS).

Chromosomal regions

To define chromosomal regions of interest, we used the high-resolution family-based full genome scans by Myers et al. [4] and Blacker et al. [5] as a basis. To transpose the positional information from the Marshfield linkage map to the NCBI sequence map, we used the mapping information of the 'UniSTS integrating markers and maps' data base (<http://www.ncbi.nlm.nih.gov/>). We took the sequence map position of the microsatellite marker with the highest signal for a linkage peak as a reference point for our interval of interest. The interval comprised the $Z^{\max-1}$ support interval of the peak marker, all sections of a peak with LOD scores >1 , and at least 5 cm upstream and downstream the peak marker assuming a linear correlation of distance between the linkage and the sequence map. For chromosome 10, we investigated an arbitrary interval comprising additional information from several fine mapping studies [8–11]. Table 2 summarizes the investigated chromosomal regions.

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Table 1 Characteristics of the investigated samples

	Group size (HCS/AD)	Age (HCS/AD)	% Females (HCS/AD)	% <i>APOE</i> $\epsilon 4$ + (HCS/AD)
Switzerland ($n=352$)	207/145	66.81 \pm 9.4/68.1 \pm 9.19	51.2/50.3	32.2/59.3
Germany ($n=117$)	77/40	71.52 \pm 8.36/80.75 \pm 6.82	40.3/65.0	25.8/53.3
Poland ($n=460$)	240/220	69.68 \pm 7.39/77.19 \pm 4.64	71.7/69.1	23.8/58.6
Belgium ($n=1200$)	464/736	58.55 \pm 15.99/75.28 \pm 8.52	54.1/66.3	30.3/54.1
Sweden ($n=361$)	169/192	72.79 \pm 5.81/77.09 \pm 7.41	62.1/62.3	29.9/60.2
Greece ($n=374$)	98/276	67.54 \pm 8.06/69.23 \pm 8.33	57.1/62.0	19.8/46.8

“Age” is age at examination for HCS, age at onset for patients (AD), and age of death in the histopathologically confirmed German sample.

Gene selection

Relation of a gene to cholesterol metabolism was assumed based on the combined information from keyword-based searches of the following databases: NCBI EntrezGene (<http://www.ncbi.nlm.nih.gov/>), GeneCards (<http://bioinfo.weizmann.ac.il/cards/index.shtml>) genmap pathways (<http://www.genmapp.org/>), Gene ontology consortium (<http://www2.ebi.ac.uk/>), The Kegg website: (<http://www.genome.ad.jp/kegg/kegg.html>). Genes that had an established or probable role in cholesterol metabolism where

checked for localization in the chromosomal regions defined above. Conversely, genes in these chromosomal regions were checked for a role in cholesterol metabolism based on the NCBI EntrezGene gene description. Although thorough, this selection process does not warrant completeness. Forty-five (45) genes fulfilled both the functional and the positional inclusion criteria (Table 2). Fourteen (14) of these genes had been investigated in previous studies with positive or negative results [12–25] (including unpublished observations). The remaining 28 genes were included in the present study (Table 3).

Table 2 Compilation of the microsatellite markers and their position in base pairs (bp) on the NCBI sequence map that define high LOD score peaks for Alzheimer’s disease (AD) in two full genome scans (Myers et al., Blacker et al.) [4, 5]

Chromosome	Marker	Reference	Sequence map position (bp)	Interval (bp)	Cholesterol-related genes in interval
1	D1S1675	Myers et al.	114541676–114541910	100000000–180000000	HMGCS2, PRKAB2, PMVK, APOA1BP, <i>FDPS</i> , APOA2, RXRG, SOAT1 ^a
3	D3S2387	Blacker et al.	1011272–1011460	0–20000000	PPARG ^a , <i>RAFTLIN</i>
4	D4S1629	Blacker et al.	158556260–158556399	150000000–170000000	
5	D5S1470	Myers et al.	32528047–32528242	22500000–47500000	PRKAA1, HMGCS1
6	D6S1018	Myers et al.	47420389–47420540	29000000–55000000	FLOT1, APOM ^a , RXRB, PPARD ^a , APOBEC2, CYP39A1
	D6S1017	Blacker et al.	41785174–41785332		ACAT2, LPA
	D6S1027	Blacker et al.	168951275–168951405	154000000–ter.	
9	D9S741	Myers et al.	24524138–24524340	15000000–45000000	
	D9S283	Blacker et al.	91604245–91604380	68000000–110000000	ABCA1 ^a
	D9S176	Myers et al.	101098125–101098298		
10	D10S1237	var.	116109983–116110380	46800000–126000000	ACF, FLJ22476 ^a , AP3M1, CH25H ^a , LIPA ^a
11	D11S968	Blacker et al.	133323661–133323811	100000000–ter.	APOA4 ^a , APOC3 ^a , APOA1 ^a , ABCG4, ZNF202, <i>ACAD8</i>
12	LOX1	Myers et al.	10202167–10216004	5000000–15000000	APOBEC1, OLR1 ^a , LRP6 ^a
14	D14S587	Blacker et al.	53436576–53436854	44000000–82000000	<i>NPC2</i>
15	D15S642	Blacker et al.	100152332–100152540	90000000–ter.	
19	D19S178	Blacker et al.	49097441–49097642	29000000–71000000	LRP3, LIPE, APOE ^a , APOC1 ^a , APOC2 ^a , NR1H2 ^a
	D19S412	Myers et al.	51702822–51702951		
21	D21S1909	Myers et al.	31455187–31455426	11000000–48000000	<i>ABCG1</i> , LSS
	D21S1440	Blacker et al.	38063497–38063658		
X	DXS8015	Myers et al.	39669120–39669305	25000000–54000000	EBP

Intervals representing peak configurations were searched for genes related to cholesterol metabolism. For chromosome 10, information from various studies (var.) [8–11] was included. Forty-five cholesterol-related genes were identified in these intervals. Genes in italics were associated with AD risk in the screening sample ($P \leq 0.05$ in Pearson’s χ^2 tests of individual SNPs or haplotypes).

^a Genes with preexisting association data were not investigated in the present study.

Table 3 Investigated genes and SNPs

Gene	SNPs 5'–3'
<i>HMGCS2</i>	<i>rs1441008</i> , <i>rs651347</i> , <i>rs668156</i> , <i>rs532208</i> , <i>rs608358</i> (<i>H_{C-T-G-G}</i> , OR=1.77, 95% CI=1.05–2.97, $\chi^2=4.71$, $P=0.03$)
PRKAB2	<i>rs1348316</i> , <i>rs2304893</i>
PMVK	<i>rs877343</i> , <i>rs1007170</i>
APOA1BP	<i>rs942960</i>
<i>FDPS</i>	<i>rs4971072</i> , <i>rs2297480</i> , <i>rs11264359</i> , <i>rs11264361</i> (<i>H_{A-G-T}</i> , $\chi^2=4.06$, $P=0.04$)
APOA2	<i>rs5082</i>
RXRG	<i>rs100537</i> , <i>rs285480</i> , <i>rs283690</i>
<i>RAFTLIN</i>	<i>rs6442608</i> , <i>rs6768991</i> , <i>rs1346604</i> , <i>rs6442605</i> , <i>rs1517516</i> , <i>rs2077624</i> , <i>rs1965432</i> , <i>rs6900</i> (<i>rs6768991_C</i> , OR=1.45, 95% CI=1.04–2.01, $\chi^2=4.89$, $P=0.03$)
PRKAA1	<i>rs466108</i> , <i>rs3805492</i> , <i>rs29742</i>
HMGCS1	<i>rs1548097</i> , <i>rs6814</i>
FLOT1	<i>rs15297</i> , <i>rs8233</i>
RXRB	<i>rs2076310</i> , <i>rs2072915</i>
APOBEC2	<i>rs2073016</i> , <i>rs11280</i>
CYP39A1	<i>rs3757241</i> , <i>rs3799866</i> , <i>rs3799877</i> , <i>rs699938</i>
ACAT2	<i>rs2277073</i> , <i>rs4832</i>
LPA	<i>rs1406888</i> , <i>rs1569933</i> , <i>rs3798221</i> , <i>rs1324785</i>
ACF	<i>rs3808919</i> , <i>rs4935194</i> , <i>rs10821846</i>
AP3M1	<i>rs3812639</i> , <i>rs6688</i> , <i>rs3809046</i> ,
ABCG4	<i>rs3809046</i> , <i>rs668033</i> , <i>rs3802885</i>
ZNF202	<i>rs675172</i>
<i>ACAD8</i>	<i>rs570113</i> , <i>rs561945</i> , <i>rs514417</i> (<i>H_{C-G-G}</i> , OR=0.45, 95% CI=0.22–0.93, $\chi^2=4.88$, $P=0.03$)
APOBEC1	<i>rs7316755</i> , <i>rs2302515</i>
<i>NPC2</i>	<i>rs1468503</i> , <i>rs917394</i> , <i>rs1860108</i> , <i>rs1029699</i> (<i>H_{G-C-T-T}</i> , OR=2.12, 95% CI=1.17–3.84, $\chi^2=6.23$, $P=0.01$)
LRP3	<i>rs3760890</i> , <i>rs875550</i>
LIPE	<i>rs851301</i> , <i>rs1206034</i>
<i>ABCG1</i>	<i>rs9976212</i> , <i>rs1378577</i> , <i>rs692383</i> , <i>rs3827225</i> , <i>rs225448</i> , <i>rs225378</i> , <i>rs425215</i> , <i>rs1044317</i> (<i>rs692383_G</i> , OR=1.82, 95% CI=1.28–2.58, $\chi^2=11.48$, $P=0.0007$)
LSS	<i>rs999689</i> , <i>rs2839158</i> , <i>rs2075906</i> , <i>rs2254524</i> , <i>rs2968</i>
EBP	<i>rs11091236</i>

Italicized letters indicate allelic or genotypic association of SNPs or their haplotypes, with AD assuming statistical significance at nominal P values of ≤ 0.05 in Pearson's χ^2 tests for allelic, genotypic, or haplotype association. These genes and SNPs were tested in an enlarged Swiss sample and in independent replication samples. Statistics are given only for the strongest effect of the respective gene. Odds ratios (OR) and corresponding confidence intervals (CI) refer to presence of the indicated allele or genotype of the respective SNP and to presence of the haplotype (H). Haplotype-defining alleles of the contributing SNPs are indicated from 5' to 3'.

SNP selection

Information on single nucleotide polymorphisms (SNPs) was derived from the NCBI dbSNP database. Eighty-four (84) SNPs corresponding to a mean resolution of ~10 kb, on average, ~40% of the genetic variation of the investi-

gated loci that would be covered by a set of tagging SNPs with a genotype correlation of $<90\%$ and a minor allele frequency of $>5\%$, and on average, three SNPs per gene, were selected by the following criteria. (1) High validation status: Ideally, SNPs reported by several independent sources were selected. (2) Appropriate position: Ideally, the SNPs selected for a gene covered the locus from upstream the 5' end to downstream the 3' end (the average position of the most 5' marker was 2,663 bp upstream of the start codon, and the average position of the most 3' marker was 230 bp downstream of the stop codon). (3) Informative allele frequencies: Ideally, SNPs with a reported minor allele frequency of $>10\%$ were selected. Information on linkage disequilibrium obtained from the Celera data base (SNPbrowser Version 2.0, Applera) was taken into consideration in the decision on SNP number and position. SNPs for which no reliable assay could be established, SNPs that did not show informative allelic distribution in our screening sample, and SNPs with strong distortion of Hardy–Weinberg equilibrium ($P<0.01$) in the control group of the screening sample were discarded and replaced by new markers. Further markers were also introduced where the most 5' or 3' SNPs yielded positive signals to facilitate delimitation from neighboring loci. Table 3 compiles the SNPs investigated as markers for the respective genes (more detailed information on the selected SNPs is provided in “electronic supplementary material” Table 5).

Genotyping

Genomic DNA was isolated from ethylenediaminetetraacetic acid blood using QIAamp DNA blood kits (Qiagen). Genotyping was done using the KASPar method (KBiosciences, <http://www.kbioscience.co.uk>). In the Belgian series, genotyping was done using 2 SEQUENOM mass array spectrometry multiplex assays, except for *rs3827225* and *rs692382* (direct sequencing) and *rs651347* and *rs1441008* (Pyrosequencing). On request, we will provide details on the individual assays including primers.

Statistics

To calculate Hardy–Weinberg equilibrium, to analyze linkage, and to reconstruct haplotypes based on pair-wise linkage disequilibrium (LD) between contributing SNPs, we used PowerMarker Version 3.22 (<http://www.powermarker.net>). We considered only those haplotypes for which the gametic phase could be predicted with a probability of $\geq 95\%$ in every individual without missing data on contributing SNPs. To assess association with AD, frequencies of genetic markers were compared between AD and HCS groups in Pearson's χ^2 tests (SPSS 12.0 for Windows). Statistical

significance was assumed for allelic, genotypic, or haplotype-specific associations with nominal P values of ≤ 0.05 . Corrections for multiple testing were not applied because with 29 independent genes and 88 markers, this very stringent procedure would have precluded the detection of moderate effects. Instead, positive findings in the screening sample were validated by sample enlargement and by replication trials in five independent additional samples. In the validation stage, i.e., in the enlarged Swiss sample and in the other five samples as well as in combined analyses, we applied unconditional forward and backward logistic regressions to adjust for age, sex, center (combined analyses) presence or absence of at least one *APOE* $\epsilon 4$ allele, and all SNPs and haplotypes of the respective gene (SPSS 12.0 for Windows). Statistical significance was assumed for associations with adjusted P values of ≤ 0.05 . For power analyses we used Power Calculator (<http://calculators.stat.ucla.edu/powercalc/>).

Results

The screening of 28 cholesterol-related genes with 84 SNPs in a sample of 115 AD cases and 191 HCS identified six candidate genes supported by nominal P values of ≤ 0.05 in Pearson's χ^2 tests of allelic, genotypic, or haplotype association (Table 3; on request, genotype details of all markers tested in the screening sample will be provided). Five of six genes showed P values of ≥ 0.01 (*HMGCS2*, *FDPS*, *RAFTLIN*, *ACAD8*, *NPC2*). The only gene significant at a higher level was *ABCG1* (rs692383, $P=0.0007$, allelic association).

To validate these observations, we investigated variants within *HMGCS2*, *FDPS*, *RAFTLIN*, *ACAD8*, *NPC2*, and *ABCG1* in an enlarged Swiss sample and in five independent replication samples of European origin. We genotyped 19 SNPs that either alone or as haplotypes were associated with AD risk in the screening sample.

Sample enlargement removed the initial significance of *HMGCS2*, *RAFTLIN*, and *NPC2* in the Swiss series. Association of *HMGCS2*, *FDPS*, *NPC2*, and *ABCG1* variants with AD was observed in at least one of the five replication samples on a gene basis at a significance level of $P \leq 0.05$. However, effects were contributed by different markers of the same gene (e.g., *FDPS*: H_{A-G-A} in Switzerland; H_{A-T-A} in Sweden) or had inverse directionality (e.g., *ABCG1* H_{C-G} : OR 1.65, 95% CI 1.06–2.59 in Switzerland; OR=0.67, 95% CI=0.46–0.98 in Poland) in different samples (Table 4, “electronic supplementary material” Tables 1 and 2). Exclusion of individuals <65 years of age did not significantly change the results (data not shown).

Previous to sample pooling for combined analyses, we compared sex distribution, age, and *APOE* $\epsilon 4$ prevalence

between the control groups of the six samples. Sex distribution and age showed strong center dependence ($P < 0.000001$). Notably, allelic or genotypic frequencies of some investigated SNPs (*HMGCS2*: rs651347, rs668156; *ACAD8*: rs570113, rs561945, rs514417; *ABCG1*: rs1378577) were also distributed differentially across control groups at significance levels of $P=0.05$ to $P=0.002$. This indicated that the comparability of the investigated samples may be limited and that sample admixture may produce false positive or false negative findings in combined analyses. Therefore, we excluded center-dependent genetic markers and haplotypes containing these markers from analyses in the pooled sample ($n=2864$). The analysis of all remaining SNPs and haplotypes did not produce consistent association of a locus with AD. Only markers for *NPC2* (rs1860108, rs1029699, H_{G-C-C}) were positive at a low significance level ($P > 0.01$). However, this effect was completely dependent on the observed association of these markers with AD in the small German sample, and logistic regression adjusting for age, sex, center, and *APOE* removed its significance. Exclusion of the younger participants (<65 years of age) and stratification for sex did not affect the result of the combined analysis. However, stratification for the presence or absence of at least one *APOE* $\epsilon 4$ allele produced an effect of *HMGCS2*, as rs532208 was associated with AD in the *APOE*- $\epsilon 4$ -positive stratum with the G allele as a risk factor and the C allele being protective ($n=1182$, OR=1.28, 95% CI 1.07–1.53, $\chi^2=7.03$, $P=0.008$, $P=0.004$ after correction for age, sex, center, and all other *HMGCS2* markers tested in the combined sample, Tables 5 and 6). Rs1441008 was also associated with AD in an *APOE*-dependent manner (Tables 5 and 6). Reassessment of the six individual samples revealed association of at least one *HMGCS2* marker with AD in the *APOE*- $\epsilon 4$ -positive or the *APOE*- $\epsilon 4$ -negative stratum for every sample at significance levels of $P=0.03$ – 0.002 (Table 7).

Discussion

In the present study, we have conducted a case control association study with those genes that are functionally related to cholesterol metabolism and are localized in chromosomal regions with high LOD scores for AD and are, therefore, both functional and positional candidate susceptibility genes for AD. During screening in a limited cohort of AD patients and healthy control subjects, most of the genes investigated with, on average, three SNPs were negative and were excluded from further analyses. This does not definitely exclude that these genes may be associated with AD because the statistical power of the screening process was low (the relative risk detectable with

Table 4 Markers associated with AD in the individual samples

	Switzerland (<i>n</i> =352)	Germany (<i>n</i> =117)	Poland (<i>n</i> =460)	Belgium (<i>n</i> =1,200)	Sweden (<i>n</i> =361)	Greece (<i>n</i> =374)
HMGCS2				H _{T-G-G-G} , OR=1.39, 95% CI=1.01– 1.91, $\chi^2=4.14$, <i>P</i> = 0.04 (<i>P</i> =0.02)	H _{T-G-G-G} , OR=2.15, 95% CI=1.11– 4.18, $\chi^2=5.27$, <i>P</i> = 0.02 (<i>P</i> =0.02)	H _{T-G-A-T} , OR= 0.58, 95% CI=0.35–0.98, $\chi^2=4.40$, <i>P</i> = 0.04 (<i>P</i> =0.03)
FDPS	H _{A-G-A} , $\chi^2=5.75$, <i>P</i> =0.02 (<i>P</i> =0.02)				H _{A-T-A} , OR=4.24, 95% CI=1.37– 13.16, $\chi^2=7.28$, <i>P</i> =0.007 (<i>P</i> = 0.007) rs2297480, rs11264359, rs11264361	
ACAD8	H _{A-T-A} , OR=0.48, 95% CI=0.25–0.93, $\chi^2=4.93$, <i>P</i> =0.03 (<i>P</i> =0.02)					
NPC2		H _{G-C-C-C} , OR=3.16, 95% CI=1.39–7.20, $\chi^2=7.76$, <i>P</i> =0.005 (<i>P</i> =0.05) rs1860108, rs1029699				
ABCG1	Rs692383 _G , OR=1.66, 95% CI=1.20–2.30, $\chi^2=9.26$, <i>P</i> =0.002 (<i>P</i> = 0.003) rs1378577, rs3827225, H _{A-A} ^a , H _{C-G} ^a		H _{C-G} ^a , OR=0.67, 95% CI=0.46– 0.98, $\chi^2=4.37$, <i>P</i> =0.04 (<i>P</i> =0.04)			

P values in italics refer to unadjusted Pearson's χ^2 tests. *P* values in parentheses were obtained by logistic regressions adjusting for sex, age, *APOE*, and all markers of the respective gene. Odds ratios (OR) and corresponding confidence intervals (CI) refer to presence of the indicated allele or genotype of the respective SNP and to presence of the haplotype (H). Haplotype-defining alleles of the contributing SNPs are indicated from 5' to 3' according to Table 3.

^aHaplotypes of *ABCG1* comprise only rs1378577 and rs692383.

Statistical details are given for the strongest effect. The other listed markers were positive on a lower significance level. Genotypic and haplotypic distributions are provided as “electronic supplementary material” (Tables 1 and 2)

80% power at a significance level of *P*=0.05 was 0.224 or 2.525 for a marker with a minor allele frequency of 0.1), and the markers used for the investigated genes did not capture the whole genetic variability of the respective loci in terms of

linkage and allele frequencies. Notably, recent studies have shown that even a dense set of tagging SNPs may be insufficient to definitely exclude association [26, 27]. Six genes, *HMGCS2*, *FDPS*, *RAFTLIN*, *ACAD8*, *NPC2*, and

Table 5 Genotypic distribution of the *HMGCS2* SNP rs532208 in the pooled sample stratified by presence or absence of at least one *APOE* ϵ 4 allele

rs532208	GG	GT	TT
HCS <i>APOE</i> ϵ 4 positive	80 (23.5%)	163 (47.8%)	96 (28.7%)
AD <i>APOE</i> ϵ 4 positive	238 (28.3%)	423 (50.3%)	180 (21.4%)
	OR=1.28, 95% CI 1.07–1.53, $\chi^2=7.03$, <i>P</i> =0.008, (<i>P</i> =0.004)		
HCS <i>APOE</i> ϵ 4 negative	237 (27.4%)	425 (49.1%)	203 (23.5%)
AD <i>APOE</i> ϵ 4 negative	178 (25.7%)	352 (50.8%)	163 (23.5%)
	OR=0.97, 95% CI 0.84–1.11, $\chi^2=0.24$, <i>P</i> =0.62, (<i>P</i> =0.33)		

Statistics refer to the comparison of the numbers of *G* vs *T* alleles (rs532208) between the HCS and AD group. The italicized *P* value refers to the unadjusted Pearson's χ^2 test. The *P* value in parentheses was obtained by logistic regression adjusting for sex, age, center, and all *HMGCS2* markers tested in the pooled sample.

Table 6 Genotypic distribution of the *HMGCS2* SNP rs1441008 in the pooled sample stratified by presence or absence of at least one *APOE* $\epsilon 4$ allele

rs1441008	CC	CT	TT
HCS <i>APOE</i> $\epsilon 4$ positive	29 (8.7%)	139 (41.5%)	167 (49.8%)
AD <i>APOE</i> $\epsilon 4$ positive	114 (13.5%)	362 (42.8%)	369 (43.7%)
	OR=1.23, 95% CI 1.06–1.56, $\chi^2=6.55$, $P=0.01$, ($P=0.02$)		
HCS <i>APOE</i> $\epsilon 4$ negative	128 (14.9%)	370 (43.0%)	362 (42.1%)
AD <i>APOE</i> $\epsilon 4$ negative	74 (10.8%)	321 (47.0%)	288 (42.2%)
	OR=0.91, 95% CI 0.97–1.06, $\chi^2=1.41$, $P=0.23$, ($P=0.26$)		

Statistics refer to the comparison of the numbers of C vs T alleles (rs1441008) between the HCS and AD group. The italicized P value refers to the unadjusted Pearson's χ^2 test. The P value in parentheses was obtained by logistic regression adjusting for sex, age, center, and all *HMGCS2* markers tested in the pooled sample.

ABCG1, were positive, assuming statistical significance at nominal P values of ≤ 0.05 . Only *ABCG1* was associated with AD at a substantially higher significance level.

To test if these observations were of any relevance beyond the investigated screening sample, we genotyped positive markers in an enlarged sample and in five independent Caucasian samples from different European countries. None of the markers showed a consistent effect across the investigated samples. Only stratification for the presence or absence of at least one *APOE* $\epsilon 4$ allele revealed a weak effect of *HMGCS2*. These findings argue against major effects of the investigated genetic variants on AD risk.

Significant differences between the control groups of the individual samples in age, sex distribution, and, notably, allelic and genotypic frequencies of some of the SNPs investigated in the present study limited the significance of a pooled analysis. Separate analyses of the samples

confirmed association of *HMGCS2*, *FDPS*, *NPC2*, and *ABCG1* with AD in at least one sample. However, across the samples, effects were carried by different SNPs or haplotypes, or the same markers showed inverse odds ratios. Our findings reflect the common problem of replication failure and inconsistency in case control association studies. It may be explained by false positivity of the original observation, i.e., type I errors. Population-specific effects of the investigated genes, owing to genetic background and environmental factors, may also explain inconsistencies between trials in independent samples. Differences in linkage and allele frequencies between different samples may lead to population-specific validity of genetic markers, which can explain inconsistency and inverse directionality of associations when the investigated genetic markers are not the functional variables underlying the observed effects. Moreover, differences in the distribu-

Table 7 All *HMGCS2* markers that showed *APOE*-dependent associated with AD in the individual samples

	<i>APOE</i> $\epsilon 4$ positive	<i>APOE</i> $\epsilon 4$ negative
Switzerland	H _{T-T-G-G} , $\chi^2=7.66$, $P=0.006$ ($P=0.006$)	H _{C-T-G-G} , OR=2.04, 95% CI 1.0–4.14, $\chi^2=3.93$, $P=0.05$ ($P=0.04$)
Germany		rs668156, $\chi^2=5.87$, $P=0.02$ ($P=0.02$) rs532208, OR _{GT} =8.12, 95% CI 1.95–33.73, $\chi^2=9.85$, $P=0.002$ ($P=0.02$) H _{T-G-G-T} , $\chi^2=6.87$, $P=0.009$ ($P=0.009$) H _{T-G-A-T} , $\chi^2=5.49$, $P=0.02$ ($P=0.02$)
Poland		rs651347, OR _{GT} =1.73, 95% CI 1.04–2.89, $\chi^2=4.5$, $P=0.03$ ($P=0.05$)
Belgium	rs651347, OR _T =1.48, 95% CI 1.08–2.03, $\chi^2=5.93$, $P=0.02$ ($P=0.01$) rs532208, OR _G =1.45, 95% CI 1.1–1.91, $\chi^2=6.89$, $P=0.009$ ($P=0.002$) H _{T-G-G-T} , OR=0.66, 95% CI 0.43–1.01, $\chi^2=3.7$, $P=0.05$ ($P=0.05$)	
Sweden		H _{T-G-G-G} , OR=2.55, 95% CI 1.08–6.0, $\chi^2=4.76$, $P=0.03$ ($P=0.02$)
Greece		H _{T-G-G-T} , OR=2.11, 95% CI 1.15–3.86, $\chi^2=5.91$, $P=0.02$ ($P=0.009$)

Italicized P values refer to unadjusted Pearson's χ^2 tests. P values in parentheses were obtained by logistic regressions adjusting for sex, age, and all *HMGCS2* markers. Odds ratios (OR) and corresponding confidence intervals (CI) refer to presence of the indicated allele or genotype of the respective SNP and to presence of the haplotype (H). Haplotype-defining alleles of the contributing SNPs are indicated from 5' to 3' according to Table 3. Genotypic and haplotypic distributions are provided as “electronic supplementary material” (Tables 3 and 4)

tion of factors like sex and age may affect the result of replication trials even if the investigated populations are genetically similar and share a similar environment. These explanations may also apply for the present study.

Although only at low significance levels, we found limited confirming evidence for association of *HMGCS2*, *FDPS*, *NPC2*, and *ABCG1* with AD. Therefore, these genes may be discussed separately:

HMGCS2 (GeneID: 3158, NCBI Entrez Gene database, <http://www.ncbi.nlm.nih.gov/>) encodes 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2. *HMGCS2* is a homologue of *HMGCS1*, a key enzyme in sterol biosynthesis and is localized in the mitochondrion. It is the first and regulating enzyme of the ketogenic pathway. Association of this gene with AD in *APOE* $\epsilon 4$ allele carriers from the pooled sample is in line with the *APOE* dependence of the linkage peak on chromosome 1 in the full genome scan by Myers et al. [4]. Separate analyses of the six samples consistently showed *APOE*-dependent association of *HMGCS2* markers with AD. However, across samples, these associations were observed for different markers, showed different strength and directionality, and occurred both in *APOE*- $\epsilon 4$ -positive and in *APOE*- $\epsilon 4$ -negative strata. The interpretability of these observations is limited by the small number of individuals in most of the sample strata. Pure stratification artifacts seem unlikely because *APOE*-dependent association of at least one *HMGCS2* marker with AD was observed in every sample. We assume that there may be complex epistatic interactions between *APOE* and genetic variables in LD with the investigated *HMGCS2* markers that may directly or indirectly lead to the observed effects. Other loci (*PHGDH*, *REG4*) are mapped in close vicinity to *HMGCS2*. Therefore, definite attribution of the signal to *HMGCS2* would require fine mapping of the region and possibly functional data in support of a role of *HMGCS2* in AD.

FDPS (GeneID: 2224, NCBI Entrez Gene database, <http://www.ncbi.nlm.nih.gov/>) encodes farnesyl diphosphate synthase. *FDPS* is a key enzyme in the isoprene biosynthetic pathway, which provides the cell with cholesterol. Like for *HMGCS2*, definite attribution of the signal to the gene is not possible with the present set of markers (neighboring loci: *PKLR*, *RUSC1*).

NPC2 (GeneID: 10577, NCBI Entrez Gene database, <http://www.ncbi.nlm.nih.gov/>) encodes Niemann–Pick disease type C2. *NPC2* may be involved in regulating the transport of cholesterol through the late endosomal/lysosomal system. Mutations in this gene have been associated with Niemann–Pick disease type C2 and frontal lobe atrophy. Also, here, delimitation of the signal from neighboring loci (*HBLD1*, *LTBP2*) would require further analyses.

ABCG1 (GeneID: 9619, NCBI Entrez Gene database, <http://www.ncbi.nlm.nih.gov/>) encodes ATP-binding cas-

sette transporter G1. *ABCG1* is a half-size transporter that, as a dimer, mediates cholesterol efflux via HDL particles. Association of the related genes *ABCA1* [16, 28, 29] and *ABCA2* [26, 30] with AD may be indirect support for a similar effect of *ABCG1*. Association of paralogous genes with AD has also been described for members of the *GAPD* gene family [31]. The gene encoding the *ABCG1* dimerization partner *ABCG4* was also investigated in the present study, but was not associated with AD. We were able to delineate the *ABCG1* signal in the 5' region of the gene. Therefore, it seems unlikely that it stems from LD with a different locus.

In previous studies, we and others have investigated several genes related to the metabolism of cholesterol for association with AD. Most of these studies were done with very few markers of the respective genes, and independent replication studies produced inconsistent results (The AlzGene Database, <http://www.alzgene.org>). In a more systematic approach, we have now investigated variants of hitherto uninvestigated cholesterol-related genes that are located in AD-linked chromosomal regions. The results of this study reflect the outcome of previous association studies that replication trials in independent populations yield only inconsistent confirmative evidence for initially observed associations. Of the cholesterol-related genes for which we have described, association with AD *CYP46A* [32] and *ABCA1* [16] may be quoted as examples of loci with several positive replication studies that are negative in meta-analyses because of reciprocal effects between individual populations (The AlzGene Database, <http://www.alzgene.org>). On the other hand, individual replication studies on an association of *SOAT1* [12] with AD were negative, but meta-analyses support a role of this gene in AD (The AlzGene Database, <http://www.alzgene.org>).

The genetic variants investigated in the present study are not associated with a strong and general modification of the risk for AD. However, inconsistent findings for *HMGCS2*, *FDPS*, *NPC2*, and *ABCG1* may provide some support for a role of further cholesterol-related genes in AD and may warrant fine mapping studies of these loci. We assume that identification of the functional variants that underlie associations observed for the genetic markers investigated in the present and in previous studies may help to overcome the prevalent problem of inconsistent replications. Once identified, these variants may form a cluster that, together with the *APOE* $\epsilon 2/3/4$ haplotype, may genetically link cholesterol metabolism and AD. Thus, the present study may contribute to the uncovering of the complex genetic underpinnings of this disease.

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